Transcriptomic and proteomic insights of the wine yeast biomass propagation process.

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ABSTRACT

Transcriptome and proteome profiles have been established for the commercial wine yeast strain T73 during an important industrial process: yeast biomass propagation. The data from both analyses reveal that the metabolic transition from fermentation to respiration is the most critical step in biomass propagation. We identified 177 ORFs and 56 proteins among those most expressed during the process, thus highlighting cell stress response, mitochondrial and carbohydrates metabolism as the most represented functional categories. A direct correlation between mRNA changes and protein abundance was observed for several functional categories such as tricarboxilic acid cycle proteins, heat shock proteins, chaperons and oxidative stress response-related proteins. However, we found no concordance in the transcript and proteomic levels for glycolytic proteins, which is probably due to post-translational modifications increasing the number of protein isoforms, especially at the end of biomass propagation. The correlation between protein abundance and the enzyme activities of alcohol dehydrogenase, pyruvate decarboxylase and glyceraldehyde-3-phosphate dehydrogenase was no affected by these modifications. We suggest post-translational mechanisms during biomass propagation which affect the stability of those proteins with an important role in the produced biomass' fermentative capacity.

INTRODUCTION

Saccharomyces cerevisiae is widely used in biotechnology and food industries, particularly in fermentative processes in which a dry active biomass is inoculated as a starter. The production of this dry active biomass involves a complex industrial propagation process which follows a scaling-up scheme combined to two different settings of yeast-growing conditions to reach high biomass yields. Initially yeast cells are grown in batches in high sucrose concentration molasses to favour fast fermentative growth until sugars are exhausted and the produced ethanol consumed. A fed-batch phase is then initiated by feeding the bioreactor with low sucrose concentration molasses in order to force the respiratory use of the carbon leading to a higher biomass yield. Oxygen is supplied from the beginning to allow both the metabolic transition to ethanol respiration after sugar exhaustion and the development of respiratory growth in the fed-batch phase. Selected industrial strains are now used predominantly in these areas and are the subject of increasingly intensive research. Several studies have evaluated the energetic, kinetic and yield parameters of the yeast biomass production process (Reed, 1982; Chen & Chiger, 1985; Reed & Nagodawithana, 1991; Degre, 1993). However, these industrial processes are poorly characterised from the point of view of yeast molecular adaptation to industrial adverse growth conditions. The difficulties encountered in studying yeast under real industrial conditions can be overcome by conducting bench-top trials which allow the application of molecular tools to gain a better understanding of the process. In the last few years, several studies have been carried out to analyse the complexity of the industrial biomass production process and to use this knowledge as a tool to improve wine yeast strains (Pérez-Torrado *et al*., 2005; Pérez-Torrado *et al*., 2009; Gómez-Pastor *et al*., 2010).

Functional genomics approaches, such as microarray profiling, are powerful tools for the analysis of gene expressions on a genomic scale, and provide a comprehensive view of yeast physiology (DeRisi *et al*., 1997; Gasch *et al*., 2000). Global gene expression analyses are frequently carried out for laboratory strains, and only a few studies have dealt with industrial yeasts, including wine yeasts (Pérez-Ortin *et al*., 2002; Rossignol *et al*., 2003; Novo *et al*., 2007), and no studies have focused on biomass propagation processes. Such knowledge is crucial to assist technological optimisation and to define genetic modification targets for the selection and improvement of wine yeasts. However, analyses of mRNA accumulation alone are not sufficient to describe a biological system since translational and post-translational regulation mechanisms are not taken into account. In most cases, gene expression levels reflect neither protein synthesis nor the activities that have been elicited to cope with changing environmental conditions. Therefore, a proteomic analysis under these specific industrial conditions is also necessary to gain a complete understanding of the yeast adaptations during the biomass propagation process. However, very few proteomic studies have been performed in wine yeast (Trabalzini *et al*., 2003; Zuzuarregui *et al*., 2006; Salvado *et al*., 2008; Rossignol *et al*., 2009) under specific experimental conditions that differ considerably from the industrial production of wine yeast biomass.

It is worth considering that a unique gene can produce a large number of protein variants due to alternative RNA processing and post-translational modifications such as oxidation, phosphorylation, acetylation and ubiquitination. These modifications provoke the appearance of different isoforms of the same protein with possible new functions (Pandey & Mann, 2000; Costa *et al*., 2002; Johnson, 2004). Thus, it is frequently difficult to correlate changes in gene expressions with changes in their protein levels

because these modifications can affect their presence and stability (Jansen & Gerstein, 2004).

Previous studies using stress gene marker analyses during bench-top trials of wine yeast biomass propagation have demonstrated the induction of specific stress-related genes and have enabled us to determine the environmental disturbances to which yeast cells are dynamically exposed (Pérez-Torrado *et al*., 2005; 2009; Gómez-Pastor *et al*., 2010). This approach has allowed us to establish critical time points throughout the process based on the profiles of different oxidative stress response genes (Pérez-Torrado *et al*., 2009; Gómez-Pastor *et al*., 2010). Industrial growth simulation was designed with two phases: an initial batch phase of growth on molasses followed by a second fed-batch phase under limiting sugar feed to ensure respiratory metabolism. Both phases were conducted under aeration conditions as usually performed in industries. Three relevant points were defined: the first during sugar starvation which marks the metabolic transition from fermentation to respiration in the batch phase; the second critical point is the end of the batch phase when previously produced ethanol is completely consumed; the third interesting point is the end of the fed-batch phase, this being the final product.

In this work, we analyze the changes in the global gene expression and proteomic profiles that occur in the wine yeast strain T73 during the biomass propagation process in order to understand the key adaptation mechanisms for the different metabolic transitions. We also provide new insights into the biotechnological improvement of this industrial process with potential economical benefits.

MATERIALS AND METHODS

Strain and media

S. cerevisiae industrial strain T73 (CECT 1894) isolated from Alicante (Spain) musts (Querol *et al*., 1992) and commercialised by Lallemand Inc. (Montreal, Canada) was used. This is a well-known industrial strain that has previously served as a model in several studies (Gimeno-Alcañiz & Matallana, 2001; Puig & Pérez-Ortín, 2000; Pérez-Torrado *et al*., 2005; 2009; Gómez-Pastor *et al*., 2010).

YPD liquid medium (1% Yeast extract, 2% Peptone, 2% Glucose) was used for preculture cultivation prior to industrial biomass propagation experiments.

Molasses medium (diluted to 60 g L^{-1} of sucrose for the batch phase or 100 g L^{-1} of sucrose for the fed-batch phase) was supplemented with 7.5 g L^{-1} of $(NH_4)_2SO_4$, 3.5 g L^{-1} of KH_2PO_4, 0.75 g L^{-1} of $MgSO_4.H_2O$, 10 mL L^{-1} of vitamin solution, and 1 mL L^{-1} of antifoam (Sigma St. Louis, Mo.). Molasses and mineral solutions were autoclaved separately. The vitamin solution, containing 50 mg L^{-1} of D-biotin, 1 g L^{-1} of calcium pantothenate, and 1 g L^{-1} of thiamine hydrochloride, was filter-sterilised (0.2 µm pore size) prior to use in the molasses medium.

Industrial propagation conditions

Biomass propagation experiments were designed with two growth stages, batch and fed-batch, in a BIOFLO III bioreactor (NBS, New Jersey, USA), and the technical parameters (agitation, pH and feed rate) were established as previously described (Pérez- Torrado *et al*., 2005; Pérez-Torrado *et al*., 2009; Gómez-Pastor *et al*., 2010). The bioreactor containing 2 L of sterilised molasses medium at pH 4.5 was inoculated to an initial optical density of 0.05 (OD600 = 0.05) from overnight YPD precultures incubated at 30ºC with shaking (250 rpm). During the batch phase, cells consumed all

the sucrose present in the medium using a fermentative metabolism. When the sucrose was finished (12-15 h), cells changed their metabolism to respiration, allowing the consumption of the ethanol produced until approximately 40 h of the process. During this phase, pH was allowed to vary freely between 4 and 5. When the ethanol was finished, the fed-batch phase started by feeding the reactor continuously with molasses medium by a type 501 peristaltic pump (Watson-Marlow, Falmouth, United Kingdom) at the desired flow rate, avoiding fermentative metabolism. During the fed-batch phase, the reactor pH was maintained at 4.5 by the automatic addition of either 42.5% (v/v) H_3PO_3 or 1 M NaOH. Dissolved oxygen, measured with an electrode (Mettler-Toledo), was maintained above 20% by a PID control system that automatically modified the agitation speed between the range limits of 300 to 500 rpm. Fermentation parameters were followed as previously described (Pérez-Torrado *et al*., 2005, Pérez-Torrado *et al*., 2009). For the different analyses, cell samples were taken using a syringe washed with cold water, frozen with liquid nitrogen and stored at -80ºC. Four independent experiments were carried out.

Total RNA preparation and cDNA synthesis

Total yeast RNA was obtained from yeast cells (50 mg) by the hot phenol method (Kohrer & Domdey, 1991). cDNA was synthesized by combining 20 µg of total RNA and 1 µg of oligo (dT) (10 to 20-mer mixture, Roche) in a final volume of 10 µl, heated for 5 min at 70ºC and chilled on ice. The elongation reaction mixture consisted of dithiothreitol (0.1 M); dNTPs mixture at 10 mM each (Invitrogen); aminoallyl-dUTP 50 mM (Fermentas); RNAse inhibitor and Superscript III reverse transcriptase (200 U/mL; Invitrogen). The reaction was incubated overnight at 50ºC. Finally the enzyme was inactivated at 70ºC for 10 min and RNA was hydrolysed in NaOH (1 M) and EDTA

(0.5 M ph 8.0) at 70ºC for 15 min. Aminoallyl-cDNA (AA-cDNA) was purified using the MinElute PCR Purification Kit (Qiagen) according to the manufacturer´s instructions.

A labelling reaction was produced by incubating 1.5-2 μg of aminoallyl-cDNA with 3 μL of the Cy3 and Cy5 fluorophores (Amersham) at a basic pH (0.2 M Na_2CO_3, pH 9) for 2 hours at room temperature. Labelled cDNA was purified using the aforementioned kit.

Microarray hybridisation and analysis

We used *Saccharomyces cerevisiae* microarray slides from the Microarray Center of University Health Network (Canada), comprising 6,240 yeast ORFs. Microarrays were prehybridised for 45 min at 42ºC in 3 x SSC (0.15 M NaCl and 0.015 M sodium citrate), 0.1% (w/v) SDS and 0.1 mg/mL BSA. Prehybridised microarrays were washed twice with water, once with isopropanol and then dried by centrifugation. Before hybridisation, equal amounts of Cy5- and Cy3-labelled cDNA, 50% (v/v) formamide, 5 x SSC and 0.1% (w/v) SDS were mixed, made up to a final volume of 60 μL with water and denatured for 1 min at 95ºC. The mixture was applied to the prehybridised microarrays, covered with a Hybri-slip (60 x 24 mm; Sigma-Aldrich) and hybridisation took place for 16 hours in a humidified chamber in a water bath at 42ºC. Hybridised microarrays were washed for 5 min at 42ºC in 2 x SSC, 0.1% (w/v) SDS for 20 min at room temperature in 0.1 x SSC, 0.1% (w/v) SDS, for 6 min at room temperature in 0.1 x SSC, and for 1 min at room temperature in 0.01 x SSC. Hybridised microarrays were dried by centrifugation. After washing, microarrays were immediately scanned with an Axon 4100A scanner at a resolution of 10 μm and the data were analysed using the GenePix Pro 6.1 software package (Axon Instruments).

185 Data were ratio-based normalised and processed using Acuity 4.0 (Axon Instruments).
186 A False Discovery Rate of 5% was used to select the statistically significant data and
187 Bonferroni correction was used. Only the $\log_2$ ratios greater than a 1.5-fold variation
188 were considered. A functional group analysis was done using the GOstat and GO term
189 finder (SGD database; http://www.yeastgenome.org) online applications. The data
190 presented correspond to the average of three biological replicates.
191
192 **Protein extraction and two-dimensional gel electrophoresis**
193 Cell samples (25 mg) were collected at each time sampling for protein extraction.
194 Owing to technical limitations, we could not collect enough cells at 5 h to perform both
195 two-dimensional gel and microarray analyses, so we set the starting point at 0 h (pre-
196 inoculum) for the proteomic experiments. Cells were resuspended in 150 µL extraction
197 buffer (8 M Urea, 25 mM Tris-HCl pH 8.0), a mixture of protease inhibitors (200 µM
198 phenylmethylsulphonyl fluoride (PMSF), 20 µM TPcK, 200 µM pepstatin A) and 0.2 g
199 of glass beads. Cells were broken in the Fast Prep (MP Bio) at 5.0 m/s for 45 sec on 3
200 occasions. After centrifugation at 12000 rpm for 10 min, the supernatant was sonicated
201 and centrifuged again at 12000 rpm for 10 min. The protein concentration was
202 determined with a Nanodrop ND-1000 UV/Vis spectrophotometer. Samples were
203 diluted to 4 µg/µL in a suspension buffer (8 M Urea, 4% (w/v) 3-cholamidopropyl-
204 dimethylammonio-propanesulfonic acid (CHAPS), 50 mM dithiothreitol, a mixture of
205 ampholytes with a pH range of 3–11 (Amersham) and traces of bromophenol blue.
206 Isoelectric focusing (50-100 µg of protein) was performed in immobilised pH gradient
207 strips (3-11 NL; Amersham). Second dimension SDS-PAGE was performed on 11%
208 acrylamide gels, followed by silver staining according to the manufacturer´s instructions
209 (Amersham Biosciences). Gels were performed for each sample in triplicate.

Quantitative gel analysis and protein identification

Gels were scanned with a GS800 densitometer (Bio-Rad) and analysed with the PDQuest software (Bio-Rad). Spot detection is mainly automatic and the software allows automatic intensity normalisation between gels. The integrated intensity ratios were calculated and analysed, and significance was evaluated using a t-test. The spot relative intensities with a p-value of < 0.05 between the three replicates were considered significant.

For protein identification, a total of 26 spots showing a minimum 2-fold difference in protein abundance between the different times and with a p-value of < 0.05 were digested with trypsin and analysed in an Applied Biosystems Voyager DE PRO MALDI-TOF. Proteins were identified by peptide mass fingerprinting with MASCOT (http://www.matrixscience.com). Protein coverage for each spot in the MASCOT analysis of up to 50 percent was considered significant. Alternatively, proteins were identified by gel matching with *S. cerevisiae* two-dimensional gel electrophoresis maps available in the following databases: YPD (Yeast Proteome Database; http://www.proteome.com), YMP (Yeast Mitochondrial Proteome; http://www.biochem.oulu.fi/proteomics/ymp.html) and 2-DE *S. cerevisiae* (IPG6-12) (http://www. weihenstephan.de/blm/deg/2ddb). A functional group analysis was done using the GOstat and GO term finder (SGD database) online applications. The data presented correspond to the average of three biological replicates.

Enzymatic activities

The cell samples (15 mg) collected at the same time sampling defined for the two-dimensional gels were broken using glass beads, and were assayed as described in the respective references; alcohol dehydrogenase (Blandino et al., 1997), pyruvate

decarboxylase (Flikweert et al., 1996) and glyceraldehydes-3-phosphate dehydrogenase (McAllister and Holland 1985).

RESULTS

Transcriptional profile of the biomass propagation process

Global gene expression experiments were done from four samples during the biomass propagation process corresponding to previously observed critical time points (Pérez-Torrado *et al*., 2005, Pérez-Torrado *et al*., 2009, Gómez-Pastor *et al*., 2010) (Figure 1). A reference sample was collected at 5 h growth, that is, at the start of the exponential phase, to avoid the effects of the characteristic lag phase in the first hours of the process. Another reason for choosing this time sampling was to avoid comparisons between the samples obtained from different growth media, YPD in the preculture and molasses in the batch, as medium composition strongly affects gene expression patterns (Shima *et al*., 2005). Two samples were taken during the diauxic shift period to gain additional information on responses helping the metabolic transition, previously analyzed by individual gene expression studies developed by northern blot (Pérez-Torrado *et al*., 2009). A second sample was collected at 14 h when there was still sucrose in the medium and when the fermentative metabolism predominated. At 18 h, the time point for the third sampling, sucrose exhaustion and high ethanol content drove the diauxic shift, that is, the transition from fermentative ethanol production to ethanol respiration. The last sample was collected at 80 h, which corresponds to the end of the process and represents the state of the biomass to be dehydrated to produce ADY (active dry yeasts). Once the time sampling for the gene expression analysis was chosen, total mRNA was obtained from each sample and microarray hybridisations were performed using three biological replicates for each time point (Figure 2).

Approximately 35% of the genes expressed during the biomass propagation process were genes with unknown functions (data not shown) whose expression was significantly induced in relation to the reference sample, but did not vary further during the process. Figure 2 shows the expression ratios corresponding to the averaged log2 signal intensity among the different microarrays. A total of 177 ORFs (see Supplementary Table 1) were differentially expressed in relation to the reference sample with a p-value of < 0.05, and they can be grouped into eight functional categories: cell stress, aerobic respiration, transport, protein degradation, metabolism, NADPH production, protein folding and ribosomal proteins.

As Figure 2 depicts, the most critical point of the process takes place at 18 h, and corresponds to the metabolic transition from fermentation to respiration. The expression profile of the 18 h sample was significantly modified, and was compared with the 14 h and 80 h time points in several functional categories. Furthermore, the number of genes for the mitochondrial proteins belonging to several functional categories was seen to be induced throughout the process, and they correspond to the induced genes as follows: 7.4% at 14 h, 15.3% at 18 h and 23.1% at 80 h. In addition, the number of genes directly related to aerobic respiration also increased during the process, as follows: 5.1% at 14 h, 6.4% at 18 h and 8.8% at 80 h. These results correlate with the cellular metabolic state at each time point in the process. Despite a predominantly fermentative metabolism occurring in the first 14-15 h of growth, the presence of oxygen regulated the induction of several genes with mitochondrial functions. The highest number of genes related to mitochondrial functions and aerobic respiration was observed in the sample at 80 h and corresponds to the final product when metabolism is predominantly respiration.

Gene expression at the end of fermentative metabolism

From the beginning of the process to 14 h of growth, metabolism is predominately fermentative due to the high levels of sucrose present in the molasses. At this time point, almost all the genes included in the functional categories cellular stress response (Fig. 2, A), TCA cycle (Fig. 2, C) and electron transport chain (Fig. 2, D), were induced, and some displayed the highest expression levels (*PRX1, YGP1, RIP1, SDH2* and *ATP20*). The expression of the genes in the other functional categories mainly remained unmodified at 14 h of growth if compared to the reference sample. Indeed, only some genes of the carbohydrates metabolism and glycerol synthesis were down-regulated at the end of the fermentative phase.

Gene expression during the diauxic shift

As mentioned earlier, the gene expression pattern was seen to be substantially modified at the 18 h time point. A broad stress response was reflected by the high induction of the genes acting in the different cellular stress mechanisms. *YPG1,* relating to nutrient starvation and ethanol stress (Destruelle *et al*., 1994; Alexandre *et al*., 2001) and *SPI1*, expressed in the stationary phase and during the diauxic shift (Cardona *et al*., 2007; 2009), were strongly induced. There was also a specific oxidative stress response, as can be observed by the induction of the relevant genes of the thioredoxin and glutathione-glutaredoxin antioxidant systems, such as *TRX2, PRX1, GRX1, GRX2, OXR1, GLO1* and *GLO2*. At least the expression levels of two of these genes (*TRX2* and *GRX2*) had been previously observed by Northern blot analysis (Pérez-Torrado *et al*., 2009; Gómez-Pastor *et al*., 2010). Another result obtained that suggests the existence of oxidative stress during the diauxic shift is the elevated induction of the *GAD1* and *UGA2* genes, both of which were implicated in the glutamate cycle and oxidative stress (Coleman *et al*., 2001).

Almost all the genes belonging to the TCA cycle category were induced, and the highest gene expression levels corresponded to the *CIT1*, *IDH1*, *IDH2*, *SDH2*, *3* and *4* and *MDH1* genes. Those genes included in the functional categories electron transport chain and energy generation were also up-regulated, which indicates glucose derepression and the induction of respiration as a result of oxygen being present. Additionally, those genes implicated in the glyoxylate cycle (*ACS1*, *AGX1*, *IDP3*, *MLS1*, *CIT2* and *ICL1*) were also highly induced. As a result of fermentable sugar starvation, the *HAP4* and *SIP2* transcriptional factors were activated, leading to an increase in the expression level of the essential genes for aerobic respiration. At the same time, *PDC1*, *5* and *6* genes, *ENO1*, *ENO2* and *CDC19* were down-regulated, whereas *GLK1*, *PCK1* and *FPB1*, which codify for enzymes catalysing irreversible glycolytic steps (Yin *et al*., 1996), were up-regulated. These results indicate not only a dynamic re-routing of the metabolic fluxes to the gluconeogenic pathway, but also glucose-6-phosphate production as a biosynthetic precursor. In addition the *TPS1* gene, involved in trehalose synthesis, and *RIM11*, *GLC8* and *GLG2*, involved in glycogen metabolism, promote carbohydrate storage. Under these metabolic conditions, glucose-6-phosphate is also used for NADPH generation which is an important reduced electron carrier during exposure to oxidative stress, as reflected by the induction of several genes of the pentose phosphate pathway (*TLK2*, *SOL4* and *RKI1)*. Hexose transporters were also up-regulated as a result of fermentable sugar starvation. Another interesting up-regulated group corresponds to FMPs genes, with an unknown function, but related to the cytoplasm to mitochondria protein transport (Neupert, 1997; Schmidt *et al*., 2001).

On the other hand, a strong down-regulation of the cytoplasmic ribosomal proteins codified by the RPLs and RPSs genes occurred at 18 h, indicating protein synthesis repression in the cytoplasm during the diauxic shift. Surprisingly, the only four

mitochondrial ribosomal proteins detected were up-regulated, which correlated with a higher number of mitochondrial induced genes during the biomass production. Moreover, those genes related to protein degradation and folding showed an induction of the mRNA levels at 18 h, indicating strong protein renovation during the diauxic shift.

Gene expression at the end of biomass propagation

The fed-batch growth stage is considered a metabolically constant phase in which cells predominantly display respiratory metabolism. At the 80 h time point, cells have been grown at a limited glucose rate to avoid sugar fermentation for 40 h. They showed a quite similar gene expression pattern to that observed at 14 h, except for those genes with mitochondrial functions, despite the different metabolic conditions at both time points. In both cases, glycerol accumulation (Pérez-Torrado, 2004) was strongly repressed by the down-regulation of the *RHR2* and *HOR2* genes, which are directly involved in glycerol synthesis, and by the up-regulation of the *GUT1* and *GUT2* genes, which are directly involved in glycerol utilisation. In general, the gene expression levels became similar to those detected at the 5 h and 14 h time points. Only those genes relating to the oxidative stress response, electron transport chain and protein transport (FMP genes) remained induced until the end of the process.

The proteome of the T73 yeast strain under industrial propagation conditions

A two-dimensional gel analysis was performed at four different sampling times during the biomass propagation to achieve a broad coverage of the process (Figure 1). The four chosen samples corresponded to representative times of the wine yeast production process: at 0 h (inoculum used as a starter, starting point), at 15 h (metabolic transition), at 40 h (end of the batch phase) and at 80 h (end of the fed-batch phase). The reference sample was taken from the inoculum in order to obtain adequate protein quantities for two gel analyses. Figure 3 shows the two-dimensional gel proteome map during the biomass propagation process for the T73 industrial yeast strain. A total of 167 differently and significantly expressed spots ($p < 0.05$) were detected between the proteome in the two biological replicates. Protein identification was performed by selecting 62 spots with the highest or the most variable abundance throughout the process, and they are described in Table 1. Two identification methods were used: mass spectrometry (27 spots) and gel matching (35 spots). The identified spots correspond to 56 proteins as several spots correspond to the same protein. The 62 identified spots were grouped into six functional categories (Table 1). The most represented functional categories during the biomass propagation process were oxidative stress and mitochondrial metabolism, with 14 identified proteins, and carbohydrate metabolism, with 15 identified proteins corresponding to 21 spots.

Proteome profile during metabolic transition

At 15 h of growth, a strong induction in the proteins relating to oxidative stress was observed. The proteins showing the highest induction levels were Prx1p, Ahp1p, Ilv5p, Cor1p and Nfu1p, followed by Gtt1p and Tsa1p. Of these, three (Prx1p, Ahp1p and Tsa1p) related to the thioredoxin antioxidant system, whose relevance during yeast

biomass propagation has been previously demonstrated (Pérez-Torrado *et al*., 2009; Gómez-Pastor *et al*., 2010). Several of the proteins involved in the stress response, such as most of the chaperones for protein folding (cytoplasmic or mitochondrial) and heat-shock proteins (Ssc1p and Mif4p), were up-regulated in this growth stage. These data correlate with the gene expression pattern observed for the protein folding-related genes during the diauxic shift, thus revealing the protein renovation phenomenon in this growth stage. The ATP metabolism-related proteins and the TCA cycle proteins were also up-regulated at 15 h.

The proteins corresponding to the carbohydrates metabolism functional category were slightly induced at the 15 h time point. However, Fba1p and Eno2p were significantly repressed by 2-fold if compared to the reference sample. Only Tdh1p showed an 8-fold accumulation, which was the highest level of protein induction during the diauxic shift. No correlation was found between the transcript levels for the carbohydrate metabolism genes and protein accumulation, especially in the case of Cdc19p, Pdc1p, Eno1p and Eno2p.

Proteome profile at the end of batch phase

Between 20 h and 40 h time points of growth in molasses, metabolism was predominately oxidative due to the presence of ethanol, which was the only carbon source until the end of this batch phase, marked by ethanol exhaustion. At the 40 h time point, those proteins relating to oxidative stress slightly accumulated if compared to the reference sample, except for Ahp1p, which remained up-regulated. Most of the oxidative stress-related proteins were markedly down-regulated compared to the sample at 15 h, especially Prx1p and Ilv5p. Additionally, Pdi1p, Rip1p and Trr1p were down-regulated if compared to the reference sample. Conversely the heat shock proteins,

401 chaperons (Kar2p and Phb1p) and proteins involved in protein synthesis (Bmh1p and
402 Bmh2p) were up-regulated.
403 The proteins involved in the carbohydrate metabolism were notably induced compared
404 to time points 0 h and 15 h, especially Tdh1p, Eno1p and Pgk1p, these being the key
405 enzymes in the gluconeogenesis metabolic pathway and Ald4p which are necessary for
406 growth in ethanol.
407 Proteome profile at the end of fed-batch phase
408 After complete ethanol consumption at 40 h, growth at a limited glucose rate was based
409 on a predominately respiratory metabolism until the end of the biomass propagation
410 process. During this stage, most of the proteins related to oxidative stress were down-
411 regulated (e.g., Trr1p, Ilv5p, Gtt1p and Sod1p), as were the cell stress response genes at
412 the end of the fed-batch phase. However some mitochondrial proteins (Cor1p, Pst2p,
413 Sod2p and Rip1p) were induced if compared to the reference sample and sampling
414 times 15 h and 40 h. These results correlate with gene expression data and indicate the
415 need for increased mitochondrial biogenesis as a result of respiratory metabolism. The
416 western blot analysis of anti-Por1p (mitochondrial porin) and anti-Sod2p, both
417 mitochondrial protein markers, showed an increase in protein levels throughout the
418 biomass propagation process (Gómez-Pastor, 2010), thus supporting the mitochondrial
419 biogenesis hypothesis.
420 Furthermore, carbohydrate metabolism proteins remained up-regulated, especially
421 Tdh1p, which was the most expressed protein in the fed-batch phase, as were Eno1p
422 and Ald4p. Interestingly, Eno2p was still down-regulated after 80 h, suggesting the
423 specific relevance of the Eno1p isoform under these growth conditions. Similarly,
424 isoform Tdh1p appears to play an important role during the process, and predominates
425 the Tdh3p isoform. However, no correlation was observed between the transcriptomic

and proteomic analyses in this functional category, suggesting that the regulation for this pathway takes place at the translational level (Rossignol *et al*., 2009).

On the other hand, heat shock proteins, chaperons, the ATP metabolism-related proteins and the TCA cycle proteins were generally down-regulated as a result of metabolic stabilisation during the fed-batch phase. Similar results were obtained in the gene expression experiments, thus showing a good correlation between both analysis types. In addition, the functional category protein synthesis, represented by Bmh1p (highly up-regulated in the fed-batch phase), Bmh2p, Tef1p and Rpc6p, was strongly induced at the end of biomass propagation process. These results correlated with the gene expression data, as observed by the induction of ribosomal genes.

Figure 4 shows the results of the comparison made of several genes detected in both the transcriptomic and proteomic analyses which allowed the identification of those genes displaying parallel or similar profiles for both mRNA and protein accumulation, such as some of the genes included in the aerobic respiration category (shown in Panel A), and those genes with clearly different profiles (shown in panel B) corresponding to the carbohydrates metabolism category.

Increased number of glycolytic enzyme isoforms during biomass propagation

An interesting phenomenon observed during the wine yeast biomass production was the increase in the number of spots with a similar molecular weight but a different isoelectric point (pI), especially for the glycolytic enzymes at the 80 h time point (Figure 5). All the proteins presented in Figure 5 were identified by mass spectrometry by analysing, at least, two spots for each protein. The enzymes with the highest increases in spots number were Tdh1p, Eno1p and Hxk1p. The first two proteins seem

to be greatly involved in the biomass propagation process as described above in the proteomic profile.

The increase in spots number for several of the proteins related to glycolysis and gluconeogenesis could account for the lack of correlation between the transcriptomic and proteomic levels in the carbohydrate metabolism functional category. Different isoforms of the same protein can be produced by post-translational modifications which could modify both the protein expression and enzyme activity irrespectively of the mRNA levels. Since the number of spots increased progressively and reached a peak at the end of the fed-batch phase, this phenomenon could be caused by protein oxidation as a result of a long period under oxidative respiration metabolic conditions.

Comparison between glycolytic protein levels, enzymatic activities and gene expression

The activity of three relevant glycolytic enzymes displaying differential expression patterns (ADH, PDC and GAPDH) was analysed throughout the biomass propagation process (Figure 6). In most of cases, a direct correlation between the protein expression levels and enzyme activities was seen. Adh1p is an isoenzyme of yeast alcohol dehydrogenase required for reducing acetaldehyde to ethanol to then catalyse the last step in the fermentative pathway. ADH activity increased at the end of the batch phase and maintained its activity until the end of this process as the protein expression levels observed during biomass propagation indicate. However, the *ADH1* gene did not show a significant change in gene expression during the global gene expression analysis (Figure 2).

Conversely, PDC activity presented the highest activity at 15 h of growth when the ethanol content was maximal. However, high activity was observed between 0 h and 15

h when a fermentative metabolism predominated. Pdc1p is the main pyruvate decarboxylase isozyme and plays a key role in alcoholic fermentation, which decarboxylates pyruvate to acetaldehyde and is subjected to regulation by glucose, ethanol and autoregulation. PDC activity was seen to correlate with the Pdc1p expression levels and there was a direct correlation with the *PDC5* and *PDC6* genes expression profiles.

GAPDH activity decreased throughout the batch phase, but increased at the end of the fed-batch phase. Although Tdh1p is one of the most up-regulated proteins during the process, its contribution to global gliceraldehyde-3-phosphate activity has been described as slight. Of the total activity, Tdh3p contributes approximately 60%, whereas Tdh1p contributes 15% (McAllister & Holland, 1985). When considering this information, we can directly correlate the Tdh3p expression levels with the GAPDH activity noted during the biomass propagation process, when enzymatic activity was 2-fold higher with a limited sugar rate than in the presence of ethanol. This enzyme diverts the glycolytic flux to pyruvate production as opposed to dihydroxyacetone and glycerol production. This increase is parallel to a decreased expression of the *RHR2* and *HOR2* genes, which are involved in glycerol synthesis, and to the up-regulation of the *GUT1* and *GUT2* genes, which are involved in glycerol utilisation. However, neither the *TDH3* or the *TDH1* genes showed significant changes in gene expression during the global gene expression analysis.

DISCUSSION

Transcriptome and proteome profiles have been established for the commercialised wine yeast strain T73 during an important industrial process: yeast biomass propagation

to produce active dried wine yeast (ADWY). The gene expression analysis allowed us to group genes into eight functional categories, of which aerobic respiration (p-value 5.21e-13), cell stress (8.34e-10) and carbohydrate metabolism (3.12e-8) are the most representative categories during yeast biomass propagation. As expected, the expression level of the genes with a mitochondrial function and of those involved in aerobic respiration (*COX4, COX13, QCR7, ATP20, STF2, SDH2, SDH3, MDH1, AGX1*) increased during the process, and correlated with the predominantly respiratory metabolism during most of the biomass propagation process. We suggest that the condition settings determine an obvious need to increase mitochondrial biogenesis as a result of glucose limitation and oxygen presence by increasing the assembly of respiratory complexes and ATP synthase (Kelly & Scarpulla, 2004). Another result that supports the importance of mitochondrial biogenesis is the increased expression of several of the FMP genes involved in the cytoplasm to the mitochondria protein transport of some enzymes, such as catalase, superoxide dismutase and cytochrome C oxidase (Neupert, 1997; Petrova *et al*., 2004; Pierrel *et al*., 2007). Similar results have been obtained in proteomic analysis where the two most represented functional categories were oxidative stress and mitochondrial metabolism (Table 1).

The diauxic shift, which takes place between 14 and 18 h of growth, is apparently the most important event during the process, as observed by the strong, broad modification of the gene expression at 18 h when compared to the other time points (DeRisi *et al*., 1997). This time point is marked by the specific induction of oxidative stress response genes such as *PRX1, TRX2* and *GRX2,* as well as *GAD1,* also involved in oxidative stress since the *gad1*$^{-}$ mutants showed increased H_2O_2 sensitivity in *S. cerevisiae*. This result indicates that the glutamate cycle could act as a redox buffering system during the diauxic shift. The importance of a proper oxidative stress response during yeast biomass

propagation has been previously demonstrated by our group as *TRX2* gene overexpression in a T73 leads to increased biomass yield production and improves the produced biomass' fermentative capacity (Pérez-Torrado *et al*., 2005; 2009; Gómez-Pastor *et al*., 2010). In a good correlation, we observed the up-regulation of Prx1p, Ahp1 and Tsa1p, which are directly related to the antioxidant thioredoxin system (Garrido & Grant, 2002; Grant, 2001; Toledano *et al*., 2003). From these results, other highly expressed genes apart from *TRX2*, such as *PRX1*, may be proposed as a new target for genetic manipulation for the purpose of obtaining further improvements in biomass propagation. In addition, several classes of genes, such as the cytochrome C-related genes and those involved in the TCA/glyoxylate cycle and carbohydrate storage, were co-ordinately induced by glucose exhaustion (DeRisi *et al*., 1997).

On the other hand, the carbohydrate metabolism-related genes showed different expression patterns during the diauxic shift. Nonetheless, the up-regulation of the key enzymes involved in irreversible steps of the glycolytic pathway (*GLK1, PCK1, FBP1*) suggests the dynamic re-routing of the metabolic fluxes to gluconeogenesis and glucose-6-phosphate production. Under oxidative stress conditions, yeast has been described to undergo a metabolic reconfiguration by activating the pentose phosphate pathway by GAPDH activity depletion and by accumulating glucose-6-phosphate to produce NADPH (Grant, 2008). This metabolic modification is supported by the induction of the *TLK2, SOL4* and *RKI1* genes involved in the pentose phosphate pathway and by GAPDH activity decreasing during the batch phase. However, no correlation was seen for the proteins and transcripts in the glycolysis category during any stage of the biomass propagation process, suggesting that regulation takes place at the translational level of this pathway. Similar results have been obtained by Rossignol and colleagues (2009) during wine fermentation.

In the diauxic shift, a strong repression of the genes coding ribosomal proteins for both the 60S and 40S ribosomal subunits also took place, indicating a strong repression of protein synthesis. This finding coincides with the data obtained in experiments simulating the diauxic shift in YPD medium (DeRisi *et al*., 1997; Ashe *et al*., 2000), when transferring cells from media with fermentable carbon sources to non-fermentable carbon sources (Kuhn et a., 2001; Diaz *et al*., 2009), and in ethanol stress experiments (Alexandre *et al*., 2001). A possible explanation could be nutrient starvation as it can relate to *YGP1* gene induction (Destruelle *et al*., 1994) and to amino acid requirements (Ashe *et al*., 2000). Actually, this hypothesis is supported by the up-regulation of the *RPN8, RPN13, RPN4* and *UBC5* genes involved in protein degradation. Protein synthesis repression is associated with a lower growth rate during this period for the purpose of saving energy and of reprogramming the gene expression profile to adapt itself to new environmental conditions (Gasch *et al*., 2000).

After the diauxic shift, oxidative stress-related genes, and especially their protein levels (Prx1p, Ahp1p, Ilv5p, Pdi1p, Sod1p, Trr1p), were simultaneously down-regulated. This scenario indicates adaptation to the new condition. Most heat shock proteins, chaperons (Mge1p, Hsp60p, Ssb1p, Ssc1p) and proteins relating to ATP metabolism were specifically induced at the 15 h time point, but their level lowered during the process. The protein data for these functional categories directly correlated to the gene expression, indicating that post-transcriptional regulatory mechanisms are not so relevant for these mRNAs groups. At the end of the industrial biomass propagation, the gene expression pattern did not vary significantly if compared to the 14 h time point, while the proteomic profile showed a strong depletion in most functional categories if compared to the initial stages. The proteins with the highest expression levels were Tdh1p, which codifies a glyceraldehyde-3-phosphate dehydrogenase, and Bmh1p and

574 Bmh2p, homologues to mammalian 14-3-3 proteins involved in global protein
575 regulation at the post-transcriptional level (Ichimura *et al*., 2004; Bruckmann *et al*.,
576 2007). The *bmh1Δ* and *bmh2Δ* mutants in *S. cerevisiae* have been described to show
577 increased levels of Fba1p, Eno1p, Eno2p, Tpi1p, Pck1p, Mdh2p, Tdh1p, Tdh3p, and
578 Gpm1p, whereas the levels of several of those proteins involved in amino acid
579 biosynthesis and translation and of heat shock proteins were lower (Bruckmann *et al*.,
580 2007).
581 Our data correlate with the assumption that the *BMH* genes seem to regulate the
582 carbohydrates metabolism genes in yeast. From the proteomic point of view, the most
583 interesting phenomenon at the end of biomass propagation process is the increased
584 number of isoforms for the glycolytic enzymes, which is reflected by the increase in
585 spots number with a similar molecular weight but different pI. Similar results have been
586 previously reported by other authors in proteins like Tpi1p, Pgi1p and Hsp26p in
587 cellular ageing, industrial ethanol production and beer fermentations (Reverter-Branchat
588 *et al*., 2004; Kolkman *et al*., 2005; Cheng *et al*., 2008). Interestingly, those enzymes
589 displaying the highest increase in spots number under our experimental conditions were
590 Tdh1p, Eno1p and Hxk1p, the first two of which are sensitive to regulation by the *BMH*
591 genes. We suggest that this phenomenon may be caused by post-translational
592 modifications (e.g., oxidations, acetylations or phosphorylations) due to a long period
593 under respiratory metabolic conditions. This may explain the lack of correlation found
594 between the transcriptomic and proteomic analyses for glycolytic enzymes (Figure 3).
595 Under oxidative stress, some glycolytic proteins (Tpi1p, Tdh3p, Eno2p, and Adh1p)
596 have been described to be specifically modified by oxidation, and different isoforms
597 appear with the same molecular weight, but different pI (Ghezzi *et al*., 2002; Costa *et*
598 *al*., 2002; LeMoan *et al*., 2006), thus affecting their activity and stability. This oxidation

process could explain the loss of fermentative capacity observed in the T73 wine yeast industrial strain at the end of biomass propagation process (Gómez-Pastor *et al*., 2010).

In order to check the correlation found between glycolytic enzyme activities and their corresponding protein levels, we assayed the enzyme activities for ADH (no spot number increase), PDC (a low spot number increase) and GAPDH (a high spot number increase). We noted a direct correlation between the enzyme activity and protein levels for all the analysed activities irrespectively of the spot number increase. These results suggest that the post-translational modifications occurring in our experimental conditions could extend the range of protein functions and probably determine locations or facilitate interactions with other proteins without affecting the original activity (reviewed in Mann & Jensen, 2003).

The combination of transcript, protein and enzymatic activity analyses provides a better overview of the mechanisms involved in the metabolic adaptation to the industrial yeast biomass propagation process. The molecular-based information provided in this work will help to gain a better understanding of the wine yeast physiology under industrial conditions, to define new targets for both the genetic and technological improvements of the industrial process, and to gain basic information about the gene-to-protein information flux.

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777

778 **FIGURE LEGENDS**

779 **Figure 1**. **Biomass production kinetics of the T73 yeast strain in molasses medium.**
780 The indicated parameters correspond to biomass production (●), sucrose consumption
781 (□), ethanol production/consumption (■), O_2 saturation (△) and sucrose feed rate (----)
782 during biomass propagation. The sampling time points for the transcriptomic (⬆: 5, 14,
783 18 and 80 h) and proteomic (⇧: 0, 15, 40 and 80 h) experiments are indicated by
784 arrows. Those samples collected at 0 h and 5 h were used as references for the
785 transcriptomic and proteomic profiles, respectively.

786 **Figure 2**. **Expression profiles of the up-regulated and down-regulated genes during**
787 **the biomass propagation process at 14 h, 18 h and 80 h**. Eight functional categories
788 were identified with a significant p-value of < 0.05 among the three biological
789 replicates. Each category can be divided into other subgroups such as (A) oxidative
790 stress, (B) general stress, (C) tricarboxilic acid cycle, (D) electron transport chain and
791 energy generation, (E) transport of proteins mitochondria/cytoplasm, (F) hexoses
792 transport, metabolism of (G) lipids, (H) glyoxylate, (I) glutamate, (J) glycogen, (K)
793 glycerol and (L) carbohydrates. The cytoplasmic (M) and mitochondrial (N) ribosomal
794 genes are also presented. For the duplicated ribosomal protein mRNA genes, only the
795 data for the A allele of each gene are shown. The colour scale at the bottom represents
796 the expression ratio x-fold repressed in green and x-fold induced in red.

797 **Figure 3. Two-dimensional protein gels during biomass propagation.** (A) 0 h; (B)
798 15 h; (C) 40 h; and (D) 80 h. Proteins were visualised with silver staining. The proteins
799 identified, whose intensity varied significantly ($p < 0.05$) among the three replicates for
800 each time point, were annotated on the gel. The underlined protein names correspond to
801 the proteins identified by mass spectrometry.

Figure 4. **Correlation between the transcriptomic and proteomic analyses**. The mRNA and protein expression levels are represented by several genes identified in both the transcriptomic and proteomic analyses, and showed two different behaviours throughout the process. Panel A indicates the direct correlation between the mRNA and protein levels for the genes related to aerobic respiration and the tricarboxilic acid cycle. Panel B presents the discrepancy between the mRNA and protein levels for the genes related to carbohydrates metabolism.

Figure 5. Glycolytic enzyme isoforms increase during biomass propagation. The two-dimensional gel magnified regions of the carbohydrates metabolism proteins whose spots number increased during biomass propagation. Proteins were visualised with silver staining. A representative image of two replicates is shown.

Figure 6. **Glycolytic enzyme activities during T73 yeast biomass propagation.** Activities for ADH (alcohol dehydrogenase), PDC (pyruvate decarboxylase) and GAPDH (glyceraldehyde 3 P dehydrogenase) are provided as μmol. min^{-1}. mg $protein^{-1}$.